CO2 enrichment does not entirely ameliorate *Vachellia karroo* drought inhibition: A missing mechanism explaining savanna bush encroachment

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ABSTRACT

There is theoretical, empirical and modeled evidence that atmospheric enrichment of carbon dioxide (CO_2) is driving the conversion of open savannas to woodlands (i.e., woody thickening or bush encroachment). This study measured the impacts of available CO_2 and water limitation on seedling growth and physiology to determine how these two environmental conditions may be influencing savanna seedling establishment. We hypothesized that a model savanna tree, *Vachellia karroo*, would experience positive growth responses to increasing CO_2 and that CO_2 enrichment would partially offset slowed growth and physiological inhibition imposed by drought. To test this hypothesis, we grew *V. karroo* in open-top chambers at the Kirstenbosch Research Center (Cape Town, South Africa). The seedlings were divided equally into four CO_2 treatments: 180 ppm, 270 ppm, 400 ppm, and 800 ppm. Seedlings were grown in their CO_2 treatments for 35 days before drought was initiated. The drought was initiated by removing the irrigation from half of the pots. Leaf gas-exchange, stem water potential, leaf non-structural carbohydrate concentrations, and plant morphology were measured at drought day-30 and drought day-60. The results showed significant benefits from CO_2 enrichment to aboveground plant structures, including larger leaves and stems, when water was abundant ($p < 0.05$). CO_2-related increases to photosynthetic parameters, such as instantaneous water-use efficiency, were significantly diminished by water limitation ($p < 0.05$). Our results present the first experimental evidence of the relationship between the two driving factors structuring the large-scale spatial patterns of savanna vegetation productivity and cover. These results suggest that atmospheric enrichment of CO_2 will significantly affect aspects of plant growth and physiology, yet prolonged drought will remain an overarching determinant of savanna plant community dynamics.

1. Introduction

1.1. African savanna biome and climate change

For the past century, tree densities have increased in savannas around the world (Stevens et al., 2016; Nackley et al., 2017). The increase of woody species suggests that there has been a release from the demographic bottlenecks that maintained the grass dominance in the structurally fluctuating savanna biome (Bond and Midgley, 2012). The two major demographic bottlenecks that limit savanna tree populations are the seedling establishment in the grass layer and sapling emergence

to mature size-classes (Higgins et al., 2000). Seedling establishment is limited by a critical period after germination when radicles must elongate beyond the ephemeral soil evaporation zone into depths of more consistently available soil moisture. Sapling emergence requires growth to resistant size-classes within intervals of "top-kill" disturbance events, such as browse or fire. There is mounting theoretical (Bond and Midgley, 2000), empirical (Kgope et al., 2009; Wigley et al., 2010; Buitenwerf et al., 2012), and modelled evidence (Higgins and Scheiter, 2012; Donohue et al., 2013; Moncrieff et al., 2014) specifically suggesting that atmospheric enrichment of carbon dioxide (CO_2) is driving the conversion of open savannas to woodlands (i.e., woody thickening

or bush encroachment). Notably, woody thickening is not uniform across precipitation gradients. The distinct African savanna biomes "arid" (< 500 mm mean annual precipitation; MAP) and "mesic" (> 700 mm MAP) merge along a narrowed precipitation gradient in the south and south-east of the continent, ultimately forming a small scale vegetation mosaic separated by subtle environmental gradients (Huntley et al., 1982). Multi-decadal data on land cover changes in South African savannas suggest that bush encroachment in arid savannas is driven primarily by land-use practices, whereas woody thickening in mesic savannas cannot only be explained by land-use changes (Buitenwerf et al., 2012; Stevens et al., 2016; Skowno et al., 2017). The variable densification across arid and mesic savannas signals that soil moisture and CO_2 are likely interacting to structure the population densities of savanna trees (Devine et al., 2017).

1.2. Savanna plants and climate change

The balance between carbon gain and water loss is fundamental to plant growth and survival. Atmospheric enrichment of CO_2 influences this balance both directly and indirectly, in water-limited regions like savannas (Polley et al., 1999; Wullschleger et al., 2002). The benefits of CO_2 enrichment to plant drought tolerance include increased photosynthesis rates, water use efficiencies (WUE), and growth rates (Wand et al., 1999; West et al., 2012; Nackley et al., 2014), accumulation of storage carbohydrates (Sala et al., 2012), and even increased availability of soil moisture (Polley et al., 1999). It has been theorized that low atmospheric CO_2 concentrations may have prevented C_3 tree growth, allowing for the dominance of grass in fire driven grassland ecosystems (Bond and Midgley, 2000; Bond et al., 2003). Models and paleo-ecological records suggest that savanna tree populations were extirpated from grasslands at the last glacial maximum (LGM; CO_2 180 ppm), survived in low densities during pre-industrial years (CO_2 270 ppm), and began to expand as CO_2 levels increased in the 20th century (Scott, 1999; Higgins et al., 2000; Bond et al., 2003).

The 33% increase in atmospheric CO_2 over the past 150 years, to concentrations unprecedented during the preceding half-million years (Petit et al., 1999), represents a dramatic global change phenomenon. The physiological basis for changes in plant performance is supported by decades of research, which show how drought, CO_2 enrichment, and warming temperatures will influence global plant communities' structure, function, and distribution (Parmesan, 2006; Donohue et al., 2013). The alteration of one factor, such as increasing CO_2, does not always correspond to ameliorating effects of other environmental processes, like increased temperature (Duan et al., 2014). For example, plants in moisture-limited situations may not be able to take advantage of the CO_2 enrichment because stomatal conductance may be restricted as the plants regulate water loss over carbon gain (Duan et al., 2013). Therefore, understanding the impacts that climate change will have on traits that allow species to grow, tolerate stress, and reproduce requires studying the interactions of multiple interrelated components of climate change.

1.3. Vachellia karroo

The current study measured seedling growth and physiology to understand how CO_2 enrichment and water limitation may be interacting and influencing savanna tree establishment. *Vachellia karroo* [(Hayne) Banfi & Galasso] (formerly *Acacia karroo*; Fabaceae) is a model tree species because of its extensive range and significant increases in population densities during the 20th century, which have been asymmetric across the mesic to arid precipitation gradient (O'Connor et al., 2014). We hypothesized that *V. karroo* seedling growth would have a positive response to increasing CO_2 and that CO_2 enrichment would partially offset slowed growth and physiological inhibition imposed by drought. Our results present the first experimental evidence of the relationship between these two driving factors

that are structuring the large-scale spatial patterns of savanna vegetation productivity and cover.

2. Methods

2.1. Experimental site and plant growth conditions

Seeds of *V. karroo* were collected from three savannas across the natural range of water availability in the Eastern Cape province of South Africa. Site 1) Arid savanna, S 32° 50′ 39″ E26 06′ 42″, elev. 692 m above mean sea level (AMSL), 417 mm mean annual precipitation (MAP), 17° C mean annual temperature (MAT). Site 2) Semi-arid savanna, S 32° 40′ 21″ E 27° 41′ 26″, elev. 501 m AMSL, 707 mm MAP and 17° C MAT. Site 3) Mesic savanna, S 32° 41′ 49″ E 28° 18′ 01″, elev. 107 m AMSL, 856 mm MAP and 18° C MAT. Seeds with visible signs of insect damage were discarded. Undamaged seeds were scarified by submersion in hot water (approximately 80° C) and soaked for 24 h before they were removed from the water and planted in sand-filled germination trays. The trays were then placed on a naturally lit, open-air tables at the Kirstenbosch Research Center (South African National Biodiversity Institute, Cape Town, South Africa). An automatic irrigation system periodically misted the trays to prevent desiccation. When enough seedlings had grown at least two true leaves (approximately five cm tall) they were transferred, individually, from the trays to custom made, sand-filled, polyvinyl chloride pots. The pots diameters were 10 cm and 50 cm long (deep) so that depth would not inhibit root elongation. in situ, *V. karroo* develops a symbiotic relationship with N-fixing rhizobia bacteria. Therefore, all pots were top-dressed with approximately 300 cm^3 of sandy-loam soil gathered at the seed collection site to promote rhizobial symbiosis. The planted pots were then set in 60 cm diameter open-top chambers (OTC).

2.2. Fumigation: CO_2 treatment

The OTCs were arranged into four CO_2 treatments group pairings. The CO_2 regimes represented the LGM (180 ppm), pre-industrial (270 ppm), ambient ca 2014 CE (400 ppm) and SRES A1FI Emissions Scenario for the year 2100 CE (800 ppm). Air delivered to all OTCs was first bubbled by a lateral channel blower (SCL V3, Effepizeta, Italy) through sealed 200-L plastic drums containing 125 L of 1.25 M sodium hydroxide solution (NaOH; Protea Chemicals, South Africa). The NaOH solution removed CO_2 from the air and was refreshed every 4–14 d, as needed. Following CO_2 removal, pure CO_2 from gas cylinders (Afrox, PTY, Ltd, South Africa) was added back into the air stream before it entered the OTC. A computer program integrated with an infrared gas analyzer (LI-820, LiCOR BioSciences Inc., Lincoln, Nebraska, USA) regulated the fumigation system. This system sampled and analyzed the CO_2 concentration in each OTC every two minutes, and automatically adjusted the CO_2 flow rate to maintain the targeted chamber CO_2 concentration. There were 16 OTCs blocked in pairs, for a total of four OTC replicates per CO_2 level. Each OTC contained 18 pots; and each pot contained one seedling, resulting in 72 plants per CO_2 regime.

Chambers were fumigated for 24 h each day, beginning on 2014.05.26 (DOY 146) and continuing for the duration of the experiment (2014.10.11; DOY 284). Light intensity and air temperature were measured using pendant data loggers (HOBO Temperature/Light 64 K, Onset Computer Corp., Pocasset, MA) hung at the height of the plant canopy inside each OTC throughout the experiment. The polycarbonate greenhouse that housed all OTC had an incident photosynthetic photon flux density (PPFD) light transmittance of 85–95%.

2.3. Irrigation: H2O treatment

Seedlings were watered automatically twice daily (before dawn and after sundown) for two minutes. Liquefied fertilizer (65 g kg^{-1} N; 27 g kg^{-1} P 130 g kg^{-1} K; Stark Ayres Inc., Cape Town, RSA) was

delivered to the seedlings via micro drip emitters. Soil water content was measured weekly at three depths (6 cm, 25 cm, and 40 cm) using a dielectric probe (ThetaProbe, ML2X, Delta-T Devices, Cambridge, UK). Soil water potential was calculated using soil moisture characteristics models for sand (Campbell, 1985).

The drought was initiated by removing the micro drip emitters from ten pots in each OTC (2014.06.30; DOY 181). The other six pots in each OTC continued to be watered twice a day. The two watering treatments, drought (D) and watered (W) were applied in a stratified random pattern, to ensure seedlings from each of the three collection sites were in both water treatment groups. After 30 days without irrigation, three plants from both water treatment groups in every OTC were harvested and analyzed. After 30 more days without irrigation (day 60 of drought), three more plants from both water treatment groups in every OTC were harvested and analyzed. At drought day 60, twice daily watering resumed on the remaining four drought plants per chamber to determine if the experimental treatments affected re-sprouting.

2.4. Measurements

Harvests (H) were conducted on 2–3 plants per treatment (CO_2 x water) at four times during the experiment. H1 was conducted four days before the onset of drought on 2014.06.25-26 (DOY 176); H2 was conducted after 30 days of drought on 2014.07.28-30 (DOY 209); H3 was conducted after 60 days of drought on 2014.08.25-27 (DOY 237). Each harvest began at midday on randomly selected individuals from each collection site within each treatment combination. Soil moisture measurements and midday leaf gas-exchange measurements were collected before harvesting the selected individual. Leaf gas-exchange was measured on the uppermost fully expanded leaves with an infrared gas analyzer that was fitted with a red/blue light source and CO_2 control unit (LI-6400-02B LED; LI-COR, Lincoln, Nebraska, USA). Gas-exchange measurements were made at the treatment CO_2 concentration for each plant, ambient air temperature, incident PPFD, and flow rate of 300 $\mu mol\ m^{-2}\ s^{-1}$. Gas-exchange measurements were recorded after the leaves had 2–5 min. to stabilize in the leaf-chamber under these conditions. After gas exchange measurements were recorded the pot was disassembled, and the roots were carefully washed free of sand. Stem length and stem diameter were measured at each harvest. Stem water potential was measured with a Scholander pressure chamber (PMS instruments, Corvallis, Oregon, USA). Plants were subdivided into leaves, stem, and roots, and dried in a draft oven at 70 °C to constant weight.

2.5. Carbohydrate analysis

Non-structural carbohydrate (NSC) concentrations were measured by a chemical assay as sucrose equivalents following a protocol modified from Alcoverro et al. (1999). Total NSC concentration was calculated as the sum of starch (mg g^{-1}) and soluble sugar (mg g^{-1}) concentrations. Oven dried plant material was ground into a fine powder in a mini-mill. Three pseudo-replicates of 0.017 g were a subset of each plant part (leaves, stem, and roots) for each seedling. Sucrose was extracted in ethanol, and the ethanol was then evaporated. The remaining sugars were re-suspended in ddH_2O and were quantified spectrophotometrically at 486 nm using a 96-well microplate spectrophotometer (Thermo Scientific Multiskan Spectrum, SkanIT software 2.4.2, 2004–2007). Starch was extracted overnight with NaOH and hydrolyzed with a solution of sulphuric acid and anthrone to yield a furfural-anthrone complex, the absorbance of which was measured at 620 nm using a 96-well microplate spectrophotometer (Thermo Scientific Multiskan Spectrum, SkanIT software 2.4.2, 2004–2007).

2.6. Statistical analysis

The experiment included eight pairs of OTC with four replicates per CO_2 treatment. As previously mentioned, each OTC contained 18 pots;

and each pot contained one seedling, resulting in 72 plants per CO_2 regime. Analysis of covariance (ANCOVA) was first used to detect a statistical of the influence of seed origin on the CO_2 and water treatment for all measured variables (e.g., stem length). After checking for normality and homogeneity of variances, split-plot two-way analysis of variance (ANOVA) was used to test the main and interactive effects of CO_2 and water on all growth and physiology parameters. The OTC was treated as the whole-plot effect, the CO_2 concentration was treated as the whole-plot factor, and the water status was treated as the split-plot factor. Holm-Sidak post hoc tests were used to compare means among CO_2 and water treatments. The effects of CO_2 and water were analyzed using 95% confidence intervals. In all cases, results were considered significant if $p < 0.05$. All analyses were performed using the using R statistical software version 3.2.2 (R Development Core Team 2015; www.r-project.org)

3. Results

3.1. Effective treatment application

Analysis of the environmental data indicated significant differences between the mean chamber CO_2 concentrations (Fig. 1). Mean values ± SD for each treatment (n = 330,800) were: 228 ± 97 ppm; 304 ± 89 ppm; 419 ± 75 ppm; 756 ± 187 ppm. Monthly low temperatures were consistently 10–15 °C throughout the experiment. As the season warmed, the average high temperatures increased from 30 °C to > 40 °C (Fig. 2). Restricting irrigation induced drought, with significantly lower soil water potential measurements in the drought treatment pots compared to the watered pots at all three depths (Fig. 3). The upper soil (6 cm) dried down rapidly. In this region, the mean soil moisture potential in the drought treatment group was −6.5 MPa at H2, and close to −10 MPa at H3. At the middle depth (25 cm) moisture in the drought treatment group was −1.5 MPa at H2, and closer to -5 MPa at H3. The bottom depth (40 cm) was significantly drier than the watered treatment but remained < −2 MPa for the duration of the experiment. The watered treatment was consistent > 0.5 MPa, at all depths.

3.2. Plant responses

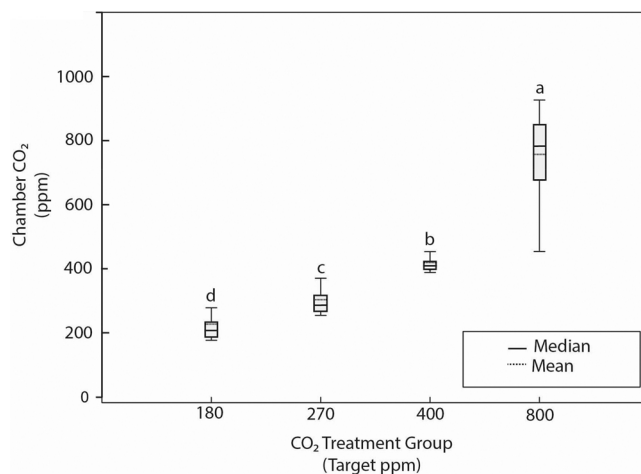The baseline harvest (H1) revealed no statistically significant

Fig. 1. CO_2 concentrations by treatment group were significantly different indicating that the seedlings experienced different growing conditions. Modified Altman whiskers extend to the 10th and 90th percentile. A Holm-Sidak pairwise comparison revealed statistically significant differences ($p < 0.001$) between all groups, denoted by the letters above the box plots. Different letters indicate significantly different mean values.
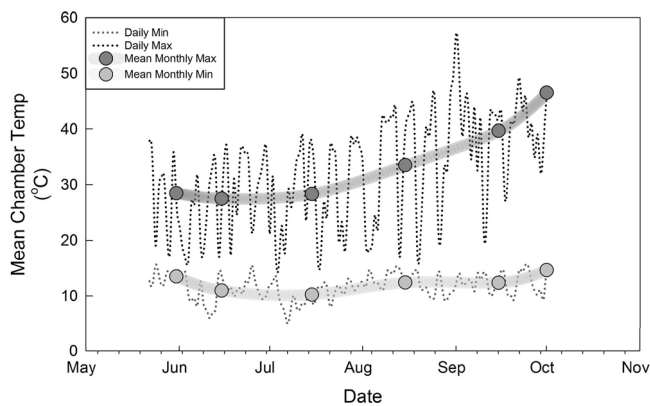
Fig. 2. Average temperatures for all 16 OTC chambers. Daily mean max and min temperatures are plotted as the dotted lines, while monthly mean max and min temperatures are plotted as the larger circles. Warming max temperatures are indicative of southern hemisphere seasonal trends.

differences between plants before application of the drought treatments ($p > 0.05$). Data for all seedlings were pooled regardless of origin because ANCOVA tests showed no significant differences between seed origin for any of the dependent variables. The drought plants had higher stem water tension, reported as greater negative MPa values (Fig. 4). At H2, 30 days into the drought, plants in the drought group had significantly lower water potentials ($p < 0.001$) than well-watered plants. At the same time, well-watered plants in the lower CO_2 treatment groups (< 400 ppm) also had significantly lower water potentials ($p < 0.01$) than well-watered plants in the higher CO_2 treatment groups (Table 1). By H3, 60 days into drought, stem water potentials were statistically different between the water treatments ($p < 0.001$). However, there was not a significant response among the different levels of CO_2 ($p > 0.05$) nor an interaction between CO_2 and water ($p > 0.05$).

Leaf gas-exchange was significantly different between the CO_2 treatment groups for both net CO_2 assimilation rates (A_{max}), and leaf transpiration (E; $p < 0.001$). A_{max} positively responded with CO_2 enrichment, and E rate was negatively related to CO_2 enrichment (Fig. 5). Both A_{max} and E rate were significantly suppressed in the drought treatment plants compared to the watered treatment group plants (Table 2). Growth responses were more variable than gas-exchange. After 30 days of drought, differences in stem diameter could not be conclusively be attributed to either of the experimental treatments (Fig. 6). Yet, after 60 days of drought, stem diameter was significantly increased by CO_2 fumigation ($p < 0.05$) and significantly decreased by water limitation ($p < 0.001$). Leaf biomass (g) was significantly increased by CO_2 enrichment ($p < 0.01$) and significantly decreased water limitation ($p < 0.001$; Fig. 6) at Harvest 2 but not Harvest 3. As the duration of the drought increased, the leaf mass of the drought plants became significantly ($p < 0.05$) smaller over time. Analysis of leaf TNC suggests that significantly higher concentrations of non-structural carbohydrates were accumulated in drought plants compared with the watered plants ($p < 0.05$; Fig. 6). Significant effects of drought were evident on stem, root and total plant biomass ($p < 0.01$). However, the CO_2 treatments did not have a significant effect on biomass allocation of these plant organs. The re-watering responses, post-drought day-60, were highly variable and not significantly related to CO_2 concentration (data not shown).

4. Discussion

Bush encroachment in savannas is occurring on a global scale (Stevens et al., 2017) in a wide variety of land management practices, and has been linked to the atmospheric enrichment of CO_2 (Bond and Midgley, 2012; Devine et al., 2017). In mesic savannas, like our sites,
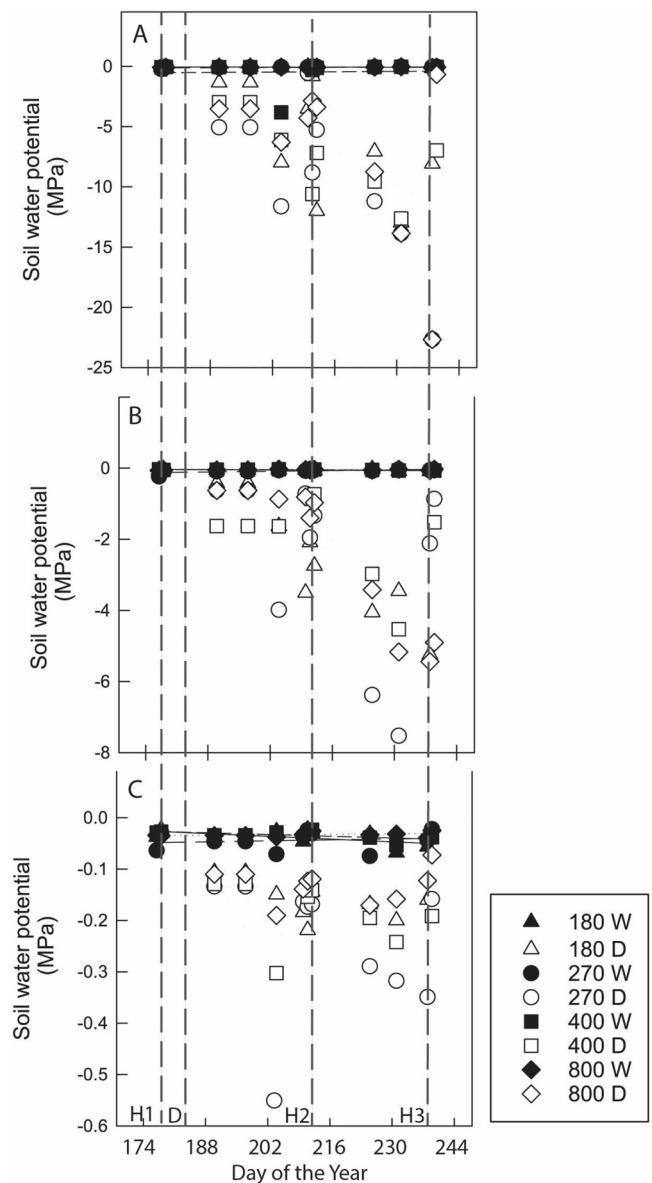

Fig. 3. Mean soil water potential throughout the experiment was significantly lower under drought treatment relative to water treatment when measured for each CO_2 x water treatment combination at depths of 6 cm (A), 25 cm (B), and 40 cm (C). Treatments are identified by target CO_2 concentration and by watered (W) or drought (D). CO_2 was not found to be significantly correlated with soil moisture at any depth at either harvest ($p > 0.05$). Significant differences between W and D were documented at all depths at both harvests ($p < 0.01$). Dashed vertical lines represent significant dates during the experiment, including drought initiation (D) and harvest dates (H1, H2 and H3).

vegetation models predict that rainfall is positively correlated with tree cover, whereas in dry African tropics (rainfall below 600 mm yr^{-1}) there is not a strong correlation between tree cover (Holmgren et al., 2013). These models are supported by remote sensing, which has documented denser encroachment in mesic savannas compared to arid savannas (Skowno et al., 2017). Our results provide experimental evidence demonstrating the additive benefits of CO_2 enrichment across a precipitation gradient and offer the first experimental evidence explaining why *V. karroo* bush encroachment has been relatively greater in mesic savannas than in arid savannas. When water was abundant, we found significant benefits to aboveground plant structures, including more leaf mass and larger stems. Increased growth of aboveground organs is ecologically relevant in the context of savanna tree
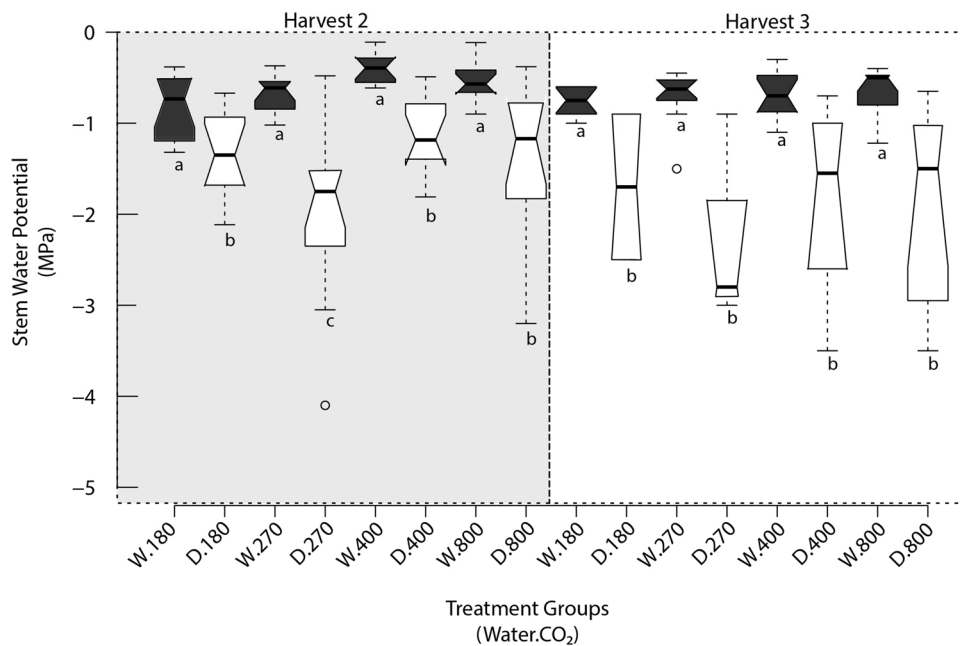
Fig. 4. Stem water potential was significantly affected by water and CO_2 treatments ($p <$ 0.01), without significant treatment interactions. ($p >$ 0.05)at Harvest 2 (left; 30 days of drought) and Harvest 3 (right; 60 days of drought). Tukey whiskers extend to data points that are less than 1.5 x IQR away from 1st or 3rd quartile. Non-overlapping notches give roughly 95% confidence that two medians differ. Lower case letters indicate mean similarity results of a Holm-sidak multiple comparison.

Table 1

Summary of mean $\pm$ SEM as well as ANOVA (P values) measured after 30 days of drought. The table is organized by CO_2 x water treatment. For example, the row labeled "180 x W" refers to watered plants within the 180 CO_2 treatment group. Significant (P ≤ 0.05) treatment effects are highlighted in bold.

	Top Soil Moisture (MPa)	Mid Soil Moisture (MPa)	Bottom Soil Moisture (MPa)	Stem Water Potential (MPa)	Stem Diameter (mm)	Leaf Mass (g)	Leaf TNC (mg g-1)
180 x W	−0.06 ± 0.02	−0.06 ± 0.01	−0.03 ± 0.01	−0.81 ± 0.1 a	1.75 ± 0.2	0.26 ± 0.04	26.8 ± 3.1
180 x D	−9.07 ± 3.26	−2.64 ± 0.81	−0.16 ± 0.03	−1.34 ± 0.1 b	1.79 ± 0.1	0.24 ± 0.04	37.5 ± 2.7
270 x W	−0.06 ± 0.02	−0.05 ± 0.01	−0.03 ± 0.01	−0.68 ± 0.1 a	2.23 ± 0.1	0.38 ± 0.06	33.6 ± 1.8
270 x D	−5.29 ± 3.03	−1.34 ± 0.35	−0.17 ± 0.03	−1.96 ± 0.3 c	2.3 ± 0.2	0.21 ± 0.02	42.9 ± 4.9
400 x W	−0.17 ± 0.12	−0.04 ± 0.01	−0.03 ± 0.01	−0.40 ± 0.1 a	2.3 ± 0.2	0.51 ± 0.09	38.2 ± 3.4
400 X D	−7.82 ± 3.32	−0.75 ± 0.21	−0.15 ± 0.03	−1.15 ± 0.1 b	2.02 ± 0.1	0.30 ± 0.05	55.3 ± 5.8
800 x W	−0.08 ± 0.02	−0.04 ± 0.01	−0.03 ± 0.01	−0.55 ± 0.1 a	2.38 ± 0.2	0.46 ± 0.09	39.0 ± 2.5
800 x D	−3.49 ± 0.97	−0.99 ± 0.31	−0.13 ± 0.01	−1.35 ± 0.2 b	2.41 ± 0.1	0.36 ± 0.05	53.9 ± 4.7
P-CO2	0.197	0.062	0.803	**0.006**	**0.002**	**0.014**	**< 0.001**
P-Water	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**	0.69	**0.004**	**< 0.001**
P-CO2 x Water	0.192	0.069	0.848	0.099	0.599	0.394	0.722

survivability because it increases the ecological success of savanna trees where top-kill is a limiting demographic bottleneck (Higgins et al., 2012). The rapid generation of aboveground biomass allows for the accelerated attainment of top-kill resistant sizes in mesic savannas. Conversely, in arid savannas seedling establishment is the limiting demographic bottleneck. Traits such as rapid radicle extension and deep rooting are important for seedling establishment in areas with low, infrequent, and irregular precipitation events during the growing season. These traits which favor plant establishment in arid regions were not significantly increased by CO_2 enrichment in our experiment, regardless of water status.

Our findings suggest that the bush encroachment observed over the past century are mediated by CO_2 enrichment, yet mitigated by soil moisture deficits. In Eastern Cape savanna systems the timing of rainfall affects competitive advantages between different plant morphologies and physiologies. For instance, plants with the C_4 photosynthesis pathway are generally considered more WUE than plants with the C_3 photosynthesis pathway. Field research in Eastern Cape savanna systems demonstrated that leaf WUE was only higher by C_4 grasses in well-watered soil and during mild drought, but differences in WUE between co-occurring C_3 and C_4 grasses were negligible under severe drought and at the beginning and end of the growing season (Taylor et al., 2014). In semi-arid and sub-humid savannas, deeper rooted perennial vegetation like *V. karroo* are also sensitive to precipitation variability,

albeit on a longer time-scale than grasses (Yang et al., 2016). Our results provide some clarity by revealing mechanisms for the disparate densification. The leaf gas-exchange results show evident increases in A_{max} and decreases in leaf transpiration (E) in response to CO_2 enrichment. Increased A_{max} and decreased E result in greater instantaneous WUE (A/E). Improved WUE has been considered a metric of fitness, a mechanism for population expansion, and one of the more consistent responses to CO_2 enrichment in C_3 plants like *V. karroo* (Wand et al., 1999; Wullschleger et al., 2002; West et al., 2007; Nackley et al., 2014). WUE is often equated with drought tolerance because the discontinuous nature of moisture availability is one of the most limiting environmental factors to plant growth in semiarid ecosystems like savannas. WUE were diminished by water limitation. In the drought treatment group, plants in the 800 ppm CO_2 group had slight assimilation rates whereas A_{max} was virtually non-existent for the 400 ppm CO_2 and the 270 ppm CO_2 groups, and was highly variable for the 180 ppm CO_2 group. While some studies have reported that elevated CO_2 may improve the water status of drought-stressed plants by reducing g_s the findings were species-dependent and may not occur under severe drought conditions (Xu et al., 2016).

Stomatal optimization theory predicts maximized WUE so that the fewest molecules of water are lost for each molecule of CO_2 absorbed (Cowan et al., 1982). The ratio of A/E is constrained by biochemical and physical factors. The conductance of water loss is driven by
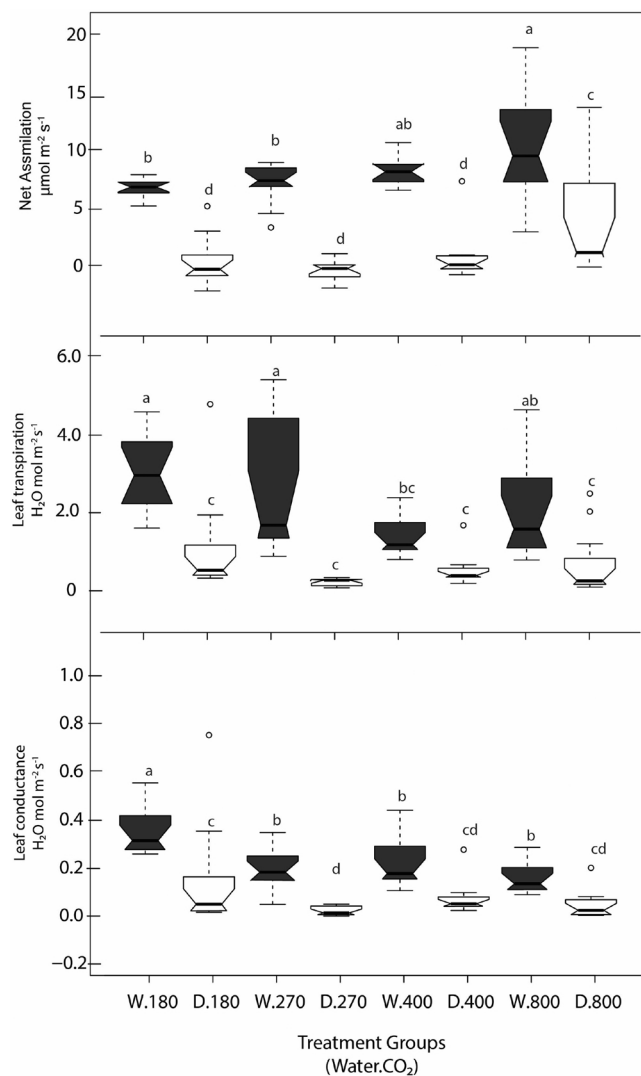
Fig. 5. Leaf gas-exchange at H3 on day 60 of drought was significantly different between the CO_2 treatment groups for both net CO_2 assimilation rates (upper panel), leaf transpiration (middle panel), and leaf conductance (lower panel). Two-way ANOVA revealed significant ($p < 0.05$) influences from both CO_2 and water treatment as well as treatment interaction for these three gas exchange metrics. Tukey whiskers extend to data points that are less than 1.5 x IQR away from 1st or 3rd quartile. Non-overlapping notches give roughly 95% confidence that two medians differ. Lowercase letters indicate mean similarity results of a Holm-sidak multiple comparison.

resistance gradients in the mesophyll, stomata, and boundary layer, whereas photosynthesis is also governed by biochemical limitations of Rubisco. Therefore the intrinsic WUE (A/E) will decline with increasing water loss. In our experiment, we observed an isohydric stomatal regulation strategy (Skelton et al., 2015), which has been described as a drought avoidance strategy. An isohydric response to drought supports the landscape level densification along precipitation gradients. When water is slightly more abundant *V. karroo* is able to optimize conversion of carbohydrates. Yet, during water deficit *V. karroo* strongly regulates stomatal conductance, even in the presence of increased CO_2. The inclusion of sub-ambient CO_2 levels (180 ppm) in our experimental design illustrates the biochemical limitations of stomatal optimization, because at 180 ppm A_{max} was significantly decreased even though conductance was significantly increased when water was abundant. Biochemical limitations to carbon assimilation lends experimental support to the theory savanna tree growth was constrained by carbohydrate assimilation during the LGM (Bond et al., 2003). WUE and stomatal

Table 2
Summary of mean ± SEM as well as ANOVA (P values) measured after 60 days of drought. The table is organized by CO_2 x water treatment. For example, the row labeled "400 x D" refers to droughted plants within the 400 CO_2 treatment group. Significant (P ≤ 0.05) treatment effects are highlighted in bold.

|	Top Soil Moisture (MPa)	Mid Soil Moisture (MPa)	Bottom Soil Moisture (MPa)	Stem Water Potential (MPa)	Stem Diameter (mm)	Leaf Mass (g)	Leaf TNC (mg g-1)	A (μmol m-2 s-1)	gs (mol m-2 s-1)	E (mmol m-2 s-1)	WUE (A/E)
180 x W	−0.11 ± 0.03	−0.08 ± 0.01	−0.06 ± 0.01	−0.78 ± 0.17	2.4 ± 0.2	0.51 ± 0.8	36.4 ± 6.5	6.74 ± 0.24	0.35 ± 0.02	2.99 ± 0.28	2.48 ± 0.2
180 x D	−8.58 ± 4.48	−4.45 ± 2.72	−0.15 ± 0.02	−1.7 ± 0.4	1.7 ± 0.1	0.08 ± 0.2	28.5 ± 7.2	0.383 ± 0.59	0.14 ± 0.06	1.05 ± 0.36	−0.24 ± 0.6
270 x W	−0.06 ± 0.01	−0.05 ± 0.01	−0.03 ± 0.01	−0.69 ± 0.17	2.4 ± 0.2	0.54 ± 0.9	24.6 ± 6.2	7.25 ± 0.49	0.20 ± 0.02	2.71 ± 0.49	3.91 ± 0.8
270 x D	−22.68 ± 0	−1.57 ± 0.39	−0.25 ± 0.06	−2.23 ± 0.34	2.0 ± 0.2	0.1 ± 0.3	40.4 ± 7.6	−0.323 ± 0.22	0.02 ± 0.01	0.23 ± 0.03	−2.18 ± 1.23
400 x W	−0.05 ± 0.01	−0.06 ± 0.01	−0.04 ± 0	−0.64 ± 0.17	2.9 ± 0.2	0.59 ± 0.8	24.4 ± 6.2	8.14 ± 0.34	0.22 ± 0.03	1.37 ± 0.15	6.47 ± 0.5
400 X D	−3.24 ± 1.54	−2.03 ± 0.78	−0.22 ± 0.03	−1.89 ± 0.21	2.1 ± 0.1	0.17 ± 0.4	44.9 ± 6.5	0.82 ± 0.68	0.08 ± 0.02	0.53 ± 0.12	0.868 ± 0.6
800 x W	−0.07 ± 0.01	−0.05 ± 0.01	−0.03 ± 0.01	−0.68 ± 0.17	2.7 ± 0.3	0.82 ± 2.1	28.5 ± 6.2	10.27 ± 1.4	0.16 ± 0.02	2.09 ± 0.38	6.08 ± 0.8
800 x D	−11.68 ± 6.35	−5.17 ± 2.42	−0.1 ± 0.01	−1.87 ± 0.26	2.2 ± 0.1	0.18 ± 0.4	47.9 ± 7.2	4.43 ± 01.48	0.05 ± 0.02	0.68 ± 0.84	6.84 ± 1.2
P-CO2	**< 0.001**	0.886	**0.002**	0.821	**0.016**	0.116	0.814	**< 0.001**	**< 0.001**	**0.014**	**< 0.001**
P-Water	**< 0.001**	**0.004**	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**	**0.014**	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**
P-CO2 x Water	**< 0.001**	0.891	**0.005**	0.772	0.405	0.499	0.128	0.67	**0.014**	**0.007**	**0.001**
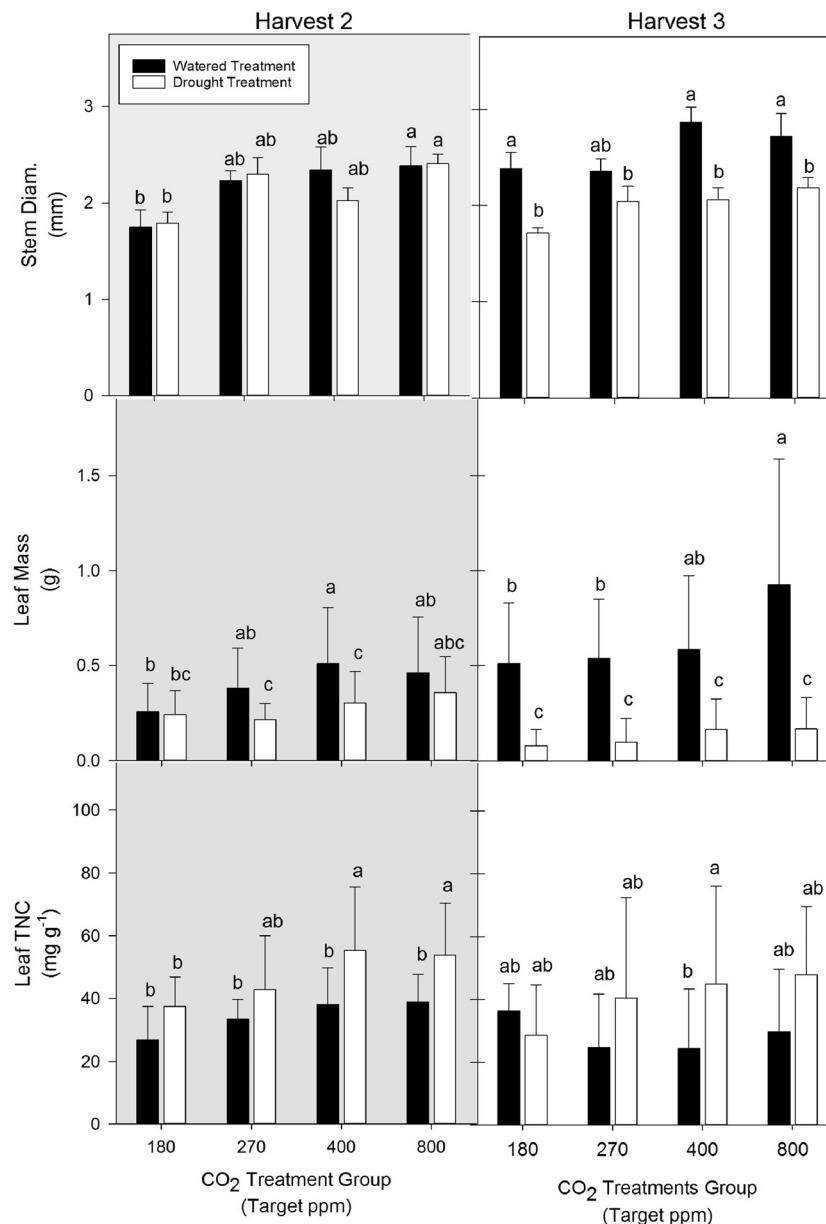
Fig. 6. Stem diameter and leaf biomass were significantly influenced by CO_2 and water at Harvest 3 ($p < 0.05$), but not at Harvest 2. Early drought significantly increased leaf total non-structural carbohydrate when CO_2 was not limited ($p < 0.05$). Lowercase letters indicate mean similarity results of a Holm-sidak multiple comparison.

optimization have physiological effects beyond photosynthesis. For instance, decreased stomatal conductance and lower transpiration rates have been shown to reduce the capacity for evaporative cooling, causing higher leaf and canopy temperatures that caused metabolic inhibition of photosynthesis (Carmo-Silva et al., 2012).

Seasonal variation in moisture can have a large effect on transpiration. Research in on trees in North American savanna systems have shown that winter precipitation falls at low intensity during times of low evaporative demand and thus percolates deeper into the soil than a similar amount of rainfall falling in summer, at high intensity, and during times of higher evaporative demand (West et al., 2008). Therefore, the timing and concentration of rainfall (not simply mean annual precipitation) must be included in models of savanna tree expansion given that precipitation patterns are predicted to become more variable in intensity and frequency. In our experiment, the increases in photosynthetic parameters such as instantaneous. At the landscape level, models of arid and semi-arid central Asian grasslands suggest that

the CO_2 fertilization effect is minimized by periods of decreased precipitation (Zhang and Ren, 2017). In semi-arid and sub-humid ecosystems, like Eastern Cape savannas, plants are less WUE and more vulnerable to extreme and variable drought than plants adapted to arid climates (Yang et al., 2016). For *V. karroo*, our results show that the drought-induced decreases in assimilation dramatically outweighed WUE associated with CO_2 enrichment.

The negative impacts of drought on carbon assimilation were also reflected in whole-plant growth and morphology. Larger aboveground plant organs were previously reported for savanna trees experiencing CO_2 enrichment (Kgope et al., 2009). Our experiment went one step further by demonstrating how drought negates any gains of CO_2 elevation. Not only were there no CO_2 stimulation effects on plant biomass in the drought treatment group, but leaf mass decreased as the drought progressed. Leaf shedding is a well-documented response to water deficits for woody and herbaceous plants and is considered a water conservation strategy for preventing desiccation and stem cavitation,

though the primary function of drought deciduousness varies among species (Wolfe et al., 2016). Studies have shown that water status mediates rising CO_2 effectiveness through the coupling of processes between gas exchange and leaf enlargement (Xu et al., 2016). Although there were fewer leaves on the drought plants on a per mass basis, the leaves from the drought treatment plants had greater total carbohydrate concentrations than plants that were watered. An increase in non-structural carbohydrate concentrations is a common, albeit not universal, response to water deficit (Sala et al., 2012). It has been suggested that leaf carbohydrate status can act as a metabolic signal although its role is not well understood (Pinheiro and Chaves, 2011). Storage of non-structural carbohydrates has been hypothesized to be an essential trait for maintenance of osmoregulation and hydraulic function during water deficit conditions (O'Brien et al., 2014). It is thought that CO_2-related reductions in stomatal conductance by plants grown in higher CO_2 concentrations will maintain higher (less negative) leaf water potentials, which could provide a form of drought resistance (Wullschleger et al., 2002; Xu et al., 2016). However, our results showed that stem water potential measurements at day 30 and day 60 of drought did not significantly respond to CO_2 enrichment, even though gs was significantly affected by the CO_2 fumigation. The response differences might be an effect of shoot-size, with larger shoots using as much water despite lowered stomatal conductance.

For *ex-situ* chamber studies it is import to reflect on whether data was collected at an appropriate stage in development to detect ecologically meaningful responses. In our experiment, the drought treatment group experienced a dry-down period in less than a month. When the first drought effects were measured (H2) the upper soil matric potential was already on average $-5\,MPa$, and mid-depths were at $-2\,MPa$. Unfortunately, no reports could be found measuring soil matric potential within the savanna range where our seeds were collected. This fundamental gap in knowledge about inter-season or inter-annual measurement of African soil matric potential reveals an opportunity for a valuable piece of basic science informing seedling development savanna tree physiology. Nonetheless, the soil moistures in this experiment can be considered appropriate for savanna species, because soil matric potentials in other arid landscapes can experience soil conditions at or below -5 MPa. Additionally, the rapid dry-down is consistent with in situ sandy savanna soils which have been documented to have an 80% reduction in volumetric water content within a similar time period (Williams and Albertson, 2004).

The fertilization effect by CO_2 enrichment was likely mitigated by the decreased ability to assimilate carbon resources necessary for growth. Plants have been classified as demonstrating isohydric or anisohydric hydraulic responses to increasing soil moisture deficits. In our experiment, *V. karroo* plants significantly decreased gs, which is a considered an isohydric-type response to limit excessive water loss and prevent cavitation from occurring in xylem conduits. This drought protection comes at the cost of reduced carbon assimilation. Plant respiration is potentially critical in determining both growth and mortality because it affects the relative amount of fixed C allocated to growth and the depletion rate of C storage during drought (McDowell, 2011). Decreased carbon assimilation for a prolonged period of drought can lead to "carbon starvation" (Sala et al., 2012) where C pools within the plant are exhausted before sufficient soil moisture is replenished and the plant's assimilation rates can exceed the respiration rates. Diminished productivity has also been attributed to decreased xylem conductivity (McDowell, 2011) as well as decreased leaf area, in the case of Asian arid lands where leaf areas of the grassland and shrubs decreased by 63% during the drought period from 1998 to 2008 (Zhang and Ren, 2017). In our experiment, we believe that carbon starvation will threaten *V. karroo* seedling survival, should the intensity and duration of drought increase during critical establishment periods.

5. Conclusion

Our results explain the disproportionate increase of tree densities in mesic savannas compared with increases in arid savanna. Given that a CO_2 effect was not detectable in the drought treatment, the mechanisms for the expansion in arid savannas remain unclear. Surviving the critical seedling establishment demographic bottleneck may be the result of narrow ecological thresholds. A rapid-dry down period means that the window of abundant available soil moisture is brief for seedling growth. In our experiment drought was imposed by restricting water for 60 days. Perhaps seedling survival and bush encroachment in arid savannas is the consequence of ephemeral, stochastic rain-fall events. The benefits from CO_2 enrichment, when water is available, could lead to the consequential amelioration of drought pressure over the course of a rainy season, which is typified by heavy rains inter-spaced between dry-spells. A delay in drought for a week, or even a few days, might allow for sufficient carbon assimilation that would result in the difference in the successful establishment of large cohorts of seedlings. In an atypically wet year, more seedlings may establish, which could develop into persistent, resistant cohort of mature-sized trees by rising atmospheric CO_2 concentrations. Field fumigation trials, or *ex-situ* experiments that test CO_2 responses across a cyclical period of drought and re-wetting that mimics the natural storm system may be able to tease out the more time-sensitive responses. Our findings suggest that drought will remain an overarching determinant of savanna plant community dynamics. However, atmospheric enrichment of CO_2 will also significantly affect aspects of plant growth and physiology. These findings further demonstrate the importance of considering multiple factors that define the dynamic ecosystem interaction.

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